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MINIREVIEW

New Frontiers in Type III Secretion Biology: the *Chlamydia* Perspective

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Members of the order *Chlamydiales* comprise a group of exquisitely evolved parasites of eukaryotic hosts that extends from single-celled amoeba to mammals. The most notable are human pathogens and include the agent of oculogenital disease *Chlamydia trachomatis*, the respiratory pathogen *C. pneumoniae*, and the zoonotic agent *C. psittaci*. All of these species are obligate intracellular bacteria that develop within parasitophorous vesicles termed inclusions. This demanding lifestyle necessitates orchestrated entry into nonphagocytic cells, creation of a privileged intracellular niche, and subversion of potent host defenses. All chlamydial genomes contain the coding capacity for a nonflagellar type III secretion system, and this mechanism has arisen as an essential contributor to chlamydial virulence. The emergence of tractable approaches to the genetic manipulation of chlamydiae raises the possibility of explosive progress in understanding this important contributor to chlamydial pathogenesis. This minireview considers challenges and recent advances that have revealed how chlamydiae have maintained conserved aspects of T3S while exploiting diversification to yield a system that exerts a fundamental role in the unique biology of *Chlamydia* species.

The nonflagellar type III secretion system (NF-T3SS) is an evolutionarily refined virulence determinant of Gram-negative bacteria where >20 proteins form an apparatus—commonly referred to as an injectisome—to accomplish the vectorial secretion and translocation of antihost effector proteins. For an excellent recent review regarding system components and the secretion process, see reference 1. Unlike other systems, the chlamydial NF-T3SS genes are not located on virulence plasmids and not arranged into pathogenicity islands. Effector genes appear to be dispersed throughout the chromosome in all *Chlamydia* species, whereas apparatus components are grouped into three major loci. The G+C content of T3SS genes resembles that of the overall genome, and there is no evidence of transposons, insertion sequence elements, or horizontal gene transfers that would suggest recent acquisition of T3S capability (2). These observations led to an early hypothesis that the chlamydial T3S may represent the primordial system from which all others arose (2, 3). More detailed phylogenetic comparisons of T3SSs have led to a model where the NF-T3SS arose from flagella by loss of flagellar genes imparting a motility function (4). Additional genes were subsequently acquired and included those for an outer membrane secretin and secreted translocon. The chlamydial T3SS would be consistent with this model, since the secretin CdsC is phylogenetically separated from other secretins (4) and contains a large, *Chlamydia*-specific N-terminal domain. Putative translocator proteins CopB and CopD are likewise highly divergent at the primary sequence level (5). Moreover, chlamydial genomes contain an apparent duplication (CopB2 and CopD2), yet biochemical evidence suggests divergence in function (6). If the evolution model is correct, however, the apparent genetic grouping of *cdcC* and other NF genes (i.e., *cdsD*) with genes of the core T3S apparatus (T3SA) strongly suggests that the chlamydial T3SS arose after the evolution of a common precursor of the NF-T3SS. Since lateral gene transfer (7) and recombination (8) are evident in modern *Chlamydia* species, it is possible that genes were acquired *en bloc* but segregated over time to reflect the current genomic distribution. Rearrangement was an ancient event, however, since overall gene organization is retained in environmental *Chlamydia*-like organisms, which diverged ca. 700 million years ago (9). Regardless of the last common ancestor, diversification of the NF-T3SS platform in *Chlamydia* species has doubtless played a role in the shaping of the chlamydial style of parasitism. It is further clear that refinement of the chlamydial NF-T3SS has continued since polymorphisms in effector proteins of clinical isolates correlate with interstrain differences in infectivity (10). In its current iteration, therefore, the chlamydial T3SS combines conserved features of T3S biology with niche-specific adaptations to directly contribute to the overall success of *Chlamydia* species as obligate intracellular parasites.

Direct investigation of the T3S mechanism in chlamydiae is cumbersome because of the obligate intracellular nature of the parasite. On the basis of genomic content analyses and apparent protein homologies, the chlamydial T3SA appears similar to other systems with respect to composition and likely function (11). Moreover, multiple protein-protein interaction studies (12–14) have indicated interactions that are consistent with models of T3SA assembly in other systems. Hence, basic aspects of the secretion mechanism are likely conserved. We will focus our discussion on the current state of knowledge regarding the chlamydial NF-T3SS and emphasize how progress has been, and will be, made in understanding this comparatively challenging system.

The most prominent distinguishing features of the chlamydial NF-T3SS arise from unique chlamydial physiology. All *Chlamydia* species possess a biphasic developmental cycle (Fig. 1) where infections are initiated when infectious particles termed elementary bodies (EBs) invade host cells. Intracellular EBs differentiate into vegetative yet noninfectious reticulate bodies (RBs) during the early cycle. RBs accumulate within a parasitophorous vacuole termed an inclusion from mid-cycle to late cycle, at which time a subset of RBs differentiate back into EBs. These EBs escape the

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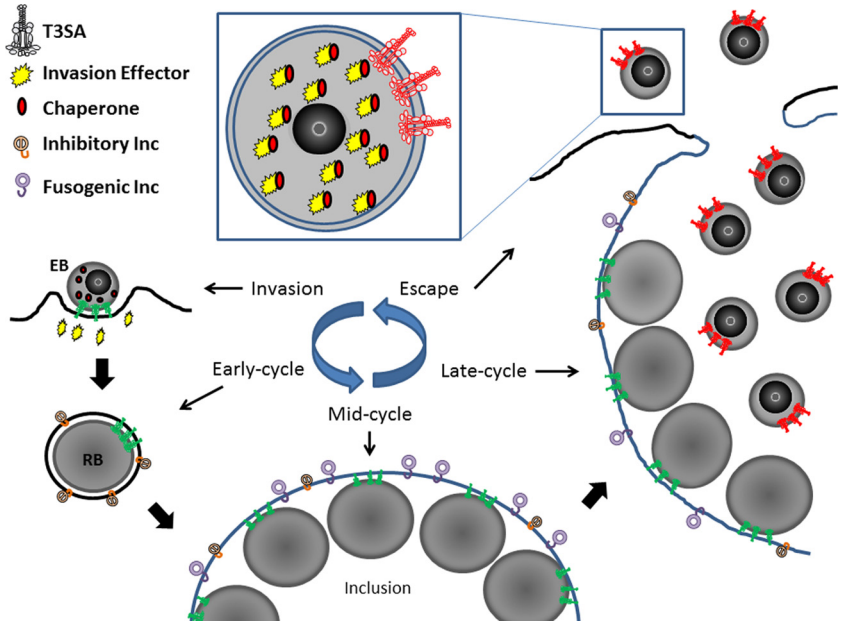

FIG 1 Working model of T3SS in chlamydial infection. Chlamydial EBs are preloaded with T3SAs, invasion-related effectors, and respective chaperones. However, the T3SA is in an inactive state (red). Contact with a host cell induces T3SA activity (depicted in green) and initiates the secretion of effectors. Early-cycle effectors, including Inc proteins that inhibit fusion with endosomal compartments, are deployed after *de novo* synthesis in RBs. Additional T3SAs are produced during the mid-cycle as chlamydiae replicate and contribute to the secretion of additional effectors that include those involved in promoting fusogenicity with host vesicular compartments. During the late cycle, cessation of T3S activity occurs during the differentiation of RBs back into EBs, presumably when RBs detach from the inclusion membrane. These systems remain off (red) during escape from the host cell and are employed during subsequent rounds of infection.

host cell and initiate subsequent rounds of infection. Given the limited metabolic activity of EBs (15), the T3SA and effectors required for invasion of epithelial cells must be prepackaged during the late-cycle conversion of RBs to EBs. Intuitively, the T3SS would be activated when an EB contacts a host cell and actively secreting injectisomes would be present on RBs. Secretion activity would be turned off in conjunction with or as a result of differentiation of RBs back to EBs following detachment from the inclusion membrane. The ability to directly test this model is limited, yet current data are consistent with the overall concept. First, it is clear that EBs contain functional T3SSs (16), and activation of effector secretion occurs rapidly upon contact with a host cell (17). These injectisomes must mediate the secretion of any early-cycle effectors, since *de novo* synthesis of T3SA genes does not occur until mid-cycle, when chlamydiae first start to divide (18, 19). Direct enumeration of presumed T3S projections on RBs has been employed in mathematical modeling analyses to provide support for a contact-dependent model of T3S activity (20). Moreover, Dumoux et al. (21) detected CdsF localization at synapse points where chlamydiae make contact with inclusion membranes. Interestingly, a quantitative proteomic approach indicates that EBs are preloaded with abundant levels of effectors and chaperones while late-cycle levels of chaperones and key apparatus components are diminished in RBs (22). The molecular mechanisms governing secretion activity remain unknown. One suggested possibility is that the chlamydial NF-T3SS is regulated in part by disulfide bonding within T3SA components. EB envelopes are highly disulfide cross-linked, and these bonds are reduced as intracellular chlamydiae differentiate into RBs (23). The chlamydial needle protein CdsF contains cysteine residues (24), a property that is unique among NF-T3SS needle proteins. Disulfide bonding within the polymerized needle correlates with the oxidation state of the chlamydial envelope and with the developmental stage (25). Hence, the disulfide bonding found in EB-localized CdsF could confer structural rigidity on this spore-like developmental form and/or have a role in the control of secretion activity.

CHAPERONE BIOLOGY

Type III secretion chaperones (T3SCs) directly bind T3S substrates and facilitate their secretion (26). In general, these dimeric, cytosol-localized proteins function by (i) protecting their substrates from degradation, (ii) maintaining their substrates in a partially unfolded secretion-competent state, (iii) preventing premature and/or unproductive protein interactions with their substrates, and (iv) directly targeting their substrates for secretion via direct interaction with selected components of the T3SA. T3SCs have been classified on the basis of their substrate specificity (27). Class I T3SCs bind single (class IA) or multiple (class IB) effector substrates, class II T3SCs bind translocon components, and class III chaperones bind needle subunit proteins. All of the chlamydial genomes analyzed to date carry genes predicted to encode examples of each class of T3SC.

Chlamydial class IA T3SCs include a heterodimeric (Scc1 and Scc4) chaperone that is specific for the N-terminal region of CopN (28). CopN is a member of the YopN/InvE/MxiC family of proteins that are found in all T3SSs and function primarily to regulate the secretion of effector and translocator proteins. Importantly, the *Chlamydia pneumoniae* CopN protein may also function as an effector protein by modulating microtubule structure within infected eukaryotic cells (29, 30). The Scc1/Scc4 T3SC has been

TABLE 1 Established invasion-related and Inc effector proteins

Effector[a]	Species range[b]	Intracellular localization	Host target(s)	Apparent domain[c]	Reference(s)
TarP (CT456)	Conserved	Cytosol	Actin	WAVE2-like actin binding domain	17, 65
CT694	*C. trachomatis*, *C. muridarum* specific	Cytosol	Ahnak	Membrane localization domain	36, 66
*Chla*OTU	*C. caviae*, *C. pneumoniae*	Cytosol	NDP52/ubiquitin	OUT domain	57
IncA (CT119)	Conserved	Inclusion membrane	Endocytic SNAREs	SNARE motif	75, 76
IncD (CT115)	*C. trachomatis*, *C. caviae*	Inclusion membrane	CERT	ND	77
IncG (CT118)	*C. trachomatis*, *C. muridarum* specific	Inclusion membrane	14-3-3-β	14-3-3 binding motif	80
CT228	*C. trachomatis*, *C. muridarum*, *C. caviae*	Inclusion membrane	Myosin phosphatase	ND	78
CT229	*C. trachomatis*, *C. muridarum* specific	Inclusion membrane	Rab4	ND	73
CT813	*C. trachomatis*, *C. muridarum* specific	Inclusion membrane	Vamp7, Vamp8	SNARE motif	75
CT850	Conserved	Inclusion membrane	Microtubule network	ND	79
Cpn0585	*C. pneumoniae* specific	Inclusion membrane	Rab1, Rab 10, Rab11	Rab binding domain	74
Cpn0517	*C. pneumoniae* specific	Inclusion membrane	Act1	ND	81

[a] Effector designations are provide as *C. trachomatis* serovar D genomic designation 2 and the formal name when appropriate.
[b] Distribution of respective genes among *C. trachomatis*, *C. muridarum*, *C. caviae*, and *C. pneumoniae*. Gene products found in all chlamydiae are designated conserved.
[c] Unknown parameters are designated not determined (ND).

shown to specifically promote the efficient secretion of CopN using a heterologous *Yersinia* T3SS. In addition, a class II T3SC, termed Scc3, binds to a C-terminal region of CopN (31), a *Chlamydia* T3SS-specific regulatory interaction that functions to reduce CopN secretion (28). Interestingly, free Scc4 (CT663) has been implicated in gene regulation through interaction with RNA polymerase (32), raising the possibility of a connection between T3S activity and genomic gene expression.

All chlamydial genomes also encode class IB or multicargo T3SCs (33). These include the Slc1 (SycE-like chaperone 1) T3SC that binds to the TarP, CT694, and CT695 effector proteins (34, 35). Slc1 has been shown to enhance the translocation of TarP via the *Yersinia* T3SS. Furthermore, TarP is present in complex with Slc1 in EBs and likely facilitates the delivery of TarP and CT694 during the invasion process (34–36). Thus, Slc1 functions to coordinate the delivery of multiple T3SS effector proteins, a function similar to class IB chaperones in other T3SSs. Other less well-characterized chlamydial class IB T3SCs include the multiple cargo secretion chaperone T3SC, which binds and stabilizes at least two Inc proteins (Cap1 and CT618) (14), and CT584, a potential T3SC that interacts with at least six T3S substrates, including CT082 (34). CT584 secretion is possible since this protein has been suggested to act as a tip complex protein (37) and is capable of binding host lipids *in vitro* (38). The presence of multiple class IB T3SCs in the *Chlamydia* T3SS suggests a possible role for these T3SCs in the establishment of a hierarchy of effector protein secretion in these organisms.

The *Chlamydia* T3SS also employs two translocator-specific or class II T3SCs termed Scc2 and Scc3 (5). The genes for these tetratricopeptide repeat-containing chaperones are homologous to the SycD T3SC of *Yersinia* and are located in close proximity to the genes that encode CopB and CopB2, respectively. Both chaperones have been shown to be capable of interacting with the *Yersinia* YopD translocator protein (5), and Scc2 coprecipitates with *C. trachomatis* CopB (5) and interacted with CopB in two-hybrid studies (14). Although the regulatory interaction of Scc3 with CopN (above) appears to be unique to the chlamydiae, class II chaperones in other T3SSs also participate in regulatory interactions that coordinate the expression and/or delivery of T3S substrates (39). The chlamydiae also employ a heterodimeric (CdsE and CdsG) class III T3SC that is specific for the CdsF needle subunit protein (24). This T3SC is similar to the *Yersinia* YscE/YscG T3SC that binds, stabilizes, and prevents premature polymerization of the YscF needle subunit protein prior to its export (40). Overall, chlamydial T3SCs are multifunctional proteins that likely play critical roles in T3SA assembly, translocon secretion and assembly, establishment of a secretion hierarchy, and the efficient translocation of effector proteins into the host cell. From a more practical standpoint, work with these chaperones has also provided an additional avenue for the identification of novel effector proteins.

EFFECTOR BIOLOGY

T3S-expressing pathogens express a diverse array of effector proteins that function by direct association, enzymatic modification, or mimicry of target host factors to facilitate molecular requirements of virulence in a given system (41). Intuitively, an obligate intracellular existence would also entail a large and intricate set of effectors. Detection of chlamydial effector proteins has been hampered by the fact that T3S substrates lack a predictable secretion signal. Effectors from other pathogens do possess consensus domains (41), yet the predictive value of these tools is limited since primary sequences are often short and require some *a priori* functional indication. Multifactorial, sequence-based analyses have been attempted for the prediction of chlamydial effectors (42, 43), but these predictions require verification because of error rates. To date, the ectopic expression and secretion of chlamydial proteins in surrogate T3SSs (44, 45) have proven to be an efficacious approach to identify candidate substrates. Since false positives are possible (46), follow-up studies typically include immunolocalization assays to detect the secretion of endogenous proteins during chlamydial infection. Given the past inability to genetically manipulate chlamydiae, subsequent functional studies have relied heavily on *in vitro* systems, protein-protein interaction studies, or ectopic expression in surrogate eukaryotic hosts. We will briefly discuss two broad classes of effectors that have emerged from ongoing studies, invasion-related and Inc class effectors. Effectors are listed in Table 1 that (i) have been shown to be secreted via T3S and by chlamydiae and (ii) have an identified host target. Additional secreted effectors such as the putative histone methyltransferase NUE (47) or the GCIP-interacting protein CT847 (48) have described host targets, yet their respective roles in chlamydial

infection remain unknown. Finally, there is an additional group of proteins that we regard as likely T3S substrates because of multiple lines of evidence. These include putative deubiquitinases ChlaDub1 and -2 (49), class I accessible protein 1 (Cap1) (50), and hypothetical proteins CT082 (34, 51), CT157 (51), CT365 (51), CT695 (34, 36), CT610 (45), CT620 (52), CT621 (52, 53), CT622 (54), CT711 (52), and CT875 (51).

Entry of chlamydiae into nonphagocytic epithelial cells is accompanied by profound alterations at the host plasma membrane (55, 56). Invasion-related effectors are presumably necessary for these orchestrated modifications. The translocated actin-recruiting phosphoprotein (TarP) and CT694 currently represent the most thoroughly characterized invasion-related effector proteins, yet we regard it likely that additional factors await discovery. In fact, Furtado et al. (57) recently described a *C. caviae* invasion-related effector designated *Chla*OTU because of the presence of an ovarian tumor (OTU) consensus domain. *Chla*OTU interacted with host NDP52 and expressed deubiquitinase activity. An ortholog is present in *C. pneumoniae* but not *C. trachomatis* and is likely to contribute to species-specific invasion requirements. Both TarP and CT694 are secreted within minutes of contact with a host cell (17, 36). *C. trachomatis* TarP recruits actin (17) and is capable of nucleating actin polymerization independently (58) or in cooperation with Arp2/3 (59). To date, neutralization by antibodies specific for the TarP actin binding domain remain the only data indicating that TarP is essential for efficient chlamydial invasion (60). *C. trachomatis* TarP is capable of being tyrosine phosphorylated (17) at entry sites by multiple host kinases (61, 62). Rac-1 is essential for *C. trachomatis* invasion (63), and Rac-1-activating factors Sos1 and Vav2 have been shown to interact with phospho-TarP (64). Since tyrosine phosphorylation is restricted to *C. trachomatis* and *C. muridarum* TarP (65), this supplementary actin manipulation capability is absent from other chlamydial species. *C. trachomatis* CT694 does not interact directly with actin but was found to interact with human Ahnak, where it appears to interfere with Ahnak-mediated actin mobilization (36). CT694 also contains a membrane localization domain similar to those found in *Yersinia* YopE, *Pseudomonas* ExoS, and *Salmonella* SptP (66), raising the possibility that CT694 may also influence actin dynamics by manipulating the activity of Rho GTPases. Combined, these findings have led to speculation that CT694 may reverse *Chlamydia*-mediated actin reorganization after the invasion process (66). Therefore, CT694 may antagonize TarP function. Given that CT694, TarP, and other putative effectors require the same secretion chaperone, it is likely that the effector interplay that has been noted in other systems (67) will also become evident in *Chlamydia* species.

Subsequent to invasion, the nascent inclusion remains segregated from endosomal compartments, traffics to the perinuclear region, and intercepts material from multivesicular bodies, lipid droplets, the endoplasmic reticulum, and the Golgi apparatus. Hence, *Chlamydia* species resemble other intracellular pathogens that reside in parasitophorous vacuoles and manipulate host membrane trafficking (68). In the chlamydial system, many of these processes are orchestrated, at least in part, by a large group of effectors termed Inc proteins. First characterized as localizing to the inclusion membrane and containing a predicted bilobed hydrophobic domain of >60 residues (69), Inc proteins are now recognized as T3S substrates (70, 71) that have a central role in the biogenesis and maintenance of the inclusion. It is important to note that the entire complement of Inc proteins is not conserved among all *Chlamydia* species. Diversity among Inc proteins likely contributes to subtle, species-specific differences in inclusion maintenance (10, 72).

Functional characterization has typically proceeded by a relatively standard set of approaches. Immunolocalization and/or biochemical studies are first employed to establish that a given Inc is localized to the inclusion membrane. Ectopically expressed, soluble Inc domains are used to identify host interaction partners via protein-protein interaction approaches such as the yeast two-hybrid system. Thus far, identified host targets include host Rabs (73, 74), soluble NSF attachment protein receptors (SNAREs) (75, 76), CERT (77), and elements of the cytoskeleton (78, 79). Additional Inc proteins apparently interact with host signaling molecules. IncG, for example, interacts with host 14-3-3β (80). While the consequences of this interaction remain unknown, IncG may function similarly to *C. pneumoniae*-specific CP0236. CP0236 binds and sequesters the signal transduction protein Act1 to interfere with proinflammatory signaling initiated by interleukin-17 (81).

A particularly interesting picture has emerged regarding *Chlamydia* IncA. Microinjection of neutralizing antibodies was first used to demonstrate a role for IncA in the homotypic fusion of inclusions (82). This finding was then confirmed by the recovery of *C. trachomatis* IncA null clinical strains that fail to undergo homotypic fusion (83). Observations that IncA could multimerize and could block chlamydial development when ectopically expressed in host cells led to the hypothesis that IncA has SNARE-like properties (84). Delevoye et al. (75) identified SNARE-like motifs in IncA, and Paumet et al. (76) subsequently employed an *in vitro* vesicle fusion assay to demonstrate that IncA inhibits the fusion of membranes requiring endocytic SNARES. Since IncA is a mid-cycle gene product, additional Inc proteins likely exist to inhibit fusion events during early-cycle development. CT813 and CT223 have been proposed as alternatives on the basis of identified SNARE-like motifs (76). While these Inc proteins interfere with endocytic membrane fusion events to avoid destruction, Rab-interacting or alternative SNARE-interacting Inc proteins likely promote vesicle fusion with nutrient-rich compartments. Hence, Inc proteins have an important role in creating and supporting a *Chlamydia*-specific privileged intracellular niche.

FUTURE PROSPECTS

Although surrogate systems and *in vitro* approaches have provided valuable insights, they do not provide the opportunity to perform definitive studies regarding the function of individual T3SS components. Bao et al. (85) have had some success in reconstituting and expressing the chlamydial T3SA in *Escherichia coli*. Although secretion activity has not been achieved, this promising approach could facilitate functional studies in a genetically tractable background. The use of chemical genetics has been attempted with chlamydiae by using small-molecule inhibitors of the NF-T3SS (86–89) and CopN (30). Inhibition of infectivity by inhibitors of the NF-T3SS is reversed by exogenous iron, and chlamydiae that are resistant to the inhibitors contain mutations in *hemG* (90), making the link to T3S activity unclear. Importantly, recent advances in chlamydial genetics may have the potential to stimulate significant progress. A variety of newly published methods laid the foundations for the genetic manipulation of *Chlamydia*. Successful mutagenesis was accomplished through expo-

sure of *C. trachomatis* to the mutagen ethyl methanesulfonate (EMS), as demonstrated by the successful isolation of a *trpB* null mutant of *C. trachomatis* (91). Furthermore, EMS mutagenesis proved to be an effective forward-genetic tool for the identification of *Chlamydia* producing inclusions with unique phenotypes or differences in virulence (92). These approaches are admittedly limited since mutations are random and any null mutation in an essential gene will not be recovered without the development of an axenic medium. However, these general approaches make it feasible to generate chlamydial strains carrying null mutations in effector genes that are not essential in the tissue culture model.

Transformation of *Chlamydia* with exogenous DNA has also been accomplished by using a variety of techniques, such as electroporation (93), $CaCl_2$ treatment (94), and the use of dendrimers (95), with each example providing additional methods to dissect protein functions. For example, a chlamydial antibiotic-selectable transformation vector was constructed that enabled the expression of green fluorescent protein in *C. trachomatis* (94). Agaisse and Derré (96) have taken a similar approach by expressing in chlamydiae fluorescent proteins that are encoded on vectors under the transcriptional control of the endogenous *C. trachomatis incD* promoter and terminator. These approaches will ultimately facilitate genetic complementation and overexpression studies to delineate the contributions of respective T3SS proteins. The functions of individual regions may also be elucidated through the expression of variants with modified or deleted domains. Methods for disrupting the expression of specific effectors may also benefit from these advances in chlamydial transformation. As suggested by the success of site-directed mutagenesis in *C. psittaci* (93), the eventual development of protocols for disrupting effector genes with antibiotic selection markers by homologous recombination should be possible. Finally, it is likely that many T3SS proteins will be essential for growth, even in a tissue culture model. Transformation could allow further study of these proteins. Dendrimer-assisted transformation of *C. trachomatis* with antisense single-stranded DNA has shown some promise in repressing chlamydial gene expression by specifically attenuating transcript levels of targeted genes (97). Hence, transient repression of essential target genes could be accomplished by using this basic approach.

With available genetic methods, confirmation of the translocation of putative effectors may be as simple as the expression of candidates fused to phosphorylatable tags (98) or enzymatic reporters (99), enabling the direct observation of successful transport into host cells by the T3SS. Given the limited size of chlamydial genomes, the eventual identification and confirmation of all effectors are possible. With a complete list of effectors and corresponding gene sequences, the deduced contributions of effector polymorphisms to virulence may finally be confirmed genetically. A more thorough characterization of *Chlamydia* effectors and the contribution of each to virulence would provide significant insight, benefitting the development of preventative and curative measures against chlamydial infection.

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